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Integrated Master's in Chemical Engineering

Fabrication of Gold Nanoparticles Inside of Unmodified Ferritins

Master's thesis

by

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Abstract

Nanoparticles, with their large range of applications in different areas are becoming a subject of growing attention in research with a promising future ahead. Gold Nanoparticles specifically, with their unique properties have become a very useful resource in many areas such as catalysis, optical sensors and medical diagnosis. Ferritins have been used to mineralize various types of nanoparticles, with the synthesis of gold nanoparticles inside ferritins being recently studied.

The objective of the proposed work is to expand a current method for the production of gold nanoparticles inside unmodified ferritins. A method will be expand in this work to also be used with the bacterial proteins Bacterioferritin (BFR) and DNA-binding proteins from starved cells (DPS) and with Horse spleen ferritin (HSFn) acting as a control. Many methods were trailed to the two ferritins (DPS and BFR) attemping to discover which method is the ideal to make encapsulated gold nanoparticles. Several challenges were overcome in order to expand this method, however some further work is needed to take advantage of the intensive research done so far.

Key words: Nanoparticles, Gold Nanoparticles, Ferritins, BFR, DPS, HSFn

Declaração

Declaro sob compromisso de honra que este documento é original e que todas as contribuições não originais foram referenciadas com identificação da fonte.

Sara Margarida Pires Rodrigues

Contents

1. Introduction 2

1.1 King's college London (BPO laboratory) 2

1.2 Background and project presentation 2

1.3 Contributions of the work 3

1.4 Thesis organisation 3

2. State of art 4

2.1 Gold nanoparticles 4

2.2 Ferritins 5

Horse spleen ferritin 6

Bacterioferritin 6

DNA binding protein from starved cells (DPS) 6

BFR and DPS 7

Nanoparticles and the ferritin family 7

2.3 Characterization techniques for the production of gold nanoparticles 8

Fast protein chromatography (FPLC) 8

Transmission electron microscope (TEM) 10

3. Experimental work 11

3.1 Description of the experimental work 12

Protein production 12

Histrap 13

Protein quantification (BCA method) 15

Production of gold nanoparticles inside horse spleen ferritin (control method) 15

Protein purification 15

4. Results and discussion 17

4.1 Quantification of the protein (BCA method) 17

4.2 Production of gold nanoparticles inside Horse spleen ferritin (control method) 17

4.3 Production of gold nanoparticles inside DPS 20

The method described to produce gold nanoparticles inside ferritins was applied in DPS the image 13 shows the SEC obtained. 21

Survability test for incubation with gold, DPS 21

Survivability test for first reduction of gold, DPS (2^{ND} STEP) 25

4.4 Production of gold nanoparticles inside BFR 29

Survability test for the incubation of gold in BFR 32

Survivability test for the first reduction of gold in BFR 35

5. Conclusions 38

5.1 Limitations and future work 39

5.2 Final appreciation 39

6. References 40

7. Additional information 41

List of Figures

Figure 1- Bacterioferritin (BFR) 6

Figure 2- DNA-binding protein from starved Cells (DPS) 7

Figure 3- Fast protein liquid chromatography (FPLC) 9

Figure 4- Schematic representation of the two step procedure to use HSFn to mineralize gold nanoparticles from a previously reported method 12

Figure 5- Schematic representation of DPS 14

Figure 6- Schematic representation of BFR 14

Figure 7- Calibration line obtained for BCA method 17

Figure 8- SEC for HSFn 18

Figure 9- SEC for HSFn with gold nanoparticles integrated 18

Figure 10- Red sample collected after the SEC 19

Figure 11- TEM image AuNPS in HSFn; TEM: HV: 120kV 19

Figure 12- Size exclusion chromatography of DPS 20

Figure 13- SEC of DPS in 0.1M AuCl4 dilute in water with AuNPs integrated 21

Figure 14- SEC of DPS in 0.01M AuCl4 dilute in water 22

Figure 15- SEC of DPS in 0.01 M AuCl4 dilute in water over 30min 23

Figure 16- SEC of DPS in 0.1 M $AuCl_4$ dilute in TRIS 23

Figure 17- SEC of DPS in 0.01 M $AuCl_4$ dilute in TRIS 24

Figure 18- SEC of DPS in 0.01M $Aucl_4$ dilute in TRIS over 30 min 24

Figure 19- SEC of DPS in 0.01M AuCl4 dilute in water with AuNPs integrated 26

Figure 20- SEC of DPS in 0.01M $AuCl_4$ dilute in water 0ver 30min with AuNPs integrated 26

Figure 21- SEC of DPS in 0.01M $AuCl_4$ dilute in TRIS with AuNPs integrated 27

Figure 22- SEC of DPS in 0.01M AuCl4 dilute in water TRIS with AuNPs integrated....27

Figure 23- SEC of DPS in the presence of ascorbic acid 28

Figure 24- SEC of DPS in the new method with NPs integrated 29

Figure 25- Size exclusion chromatography of BFR 30

Figure 26- SEC of BFR in 0.01M AuCl4 dilute in water with AuNPs integrated.....30

Figure 27- TEM image AuNPS in BFR; TEM: HV:120kV 31

Figure 28-- SEC of BFR in 0.01 M $AuCl_4$ dilute in water 33

Figure 29- SEC of BFR in 0.01 M AuCl4 dilute in water over 30 min 33

Figure 30- SEC of BFR in 0.1M $AuCl_4$ dilute in TRIS 34

Figure 31- SEC of BFR in 0.01 M AuCl4 dilute TRIS 34

Figure 32- SEC of BFR in 0.01 M AuCl4 dilute TRIS over 30 min 35

Figure 33- SEC of BFR in 0.1 AuCl4 dilute in Tris with AuNPs integrated 36

Figure 34-- TEM image of BFR in 0.01M AuCl4 dilute in TRIS 120kV 37

List of tables

Table 1- Survability test of DPS in $AuCl_4$.....22

Table 2- Results for the production of gold nanoparticles inside DPS.....25

Table 3- Survability test of BFR in $AuCl_4$.....32

Table 4- Results for the production of gold nanoparticles inside BFR.....36

Glossary

List of units

M (mol/l)	Molar Concentration
mAU	Absorbance unit from UV- visible spectroscopy
kV	Potential: voltage

List of abbreviations

BFR	Bacterioferritin
DPS	DNA-Binding Protein from Starved Cells
HSFn	Horse Spleen Ferritin
NPs	Nanoparticles
AuNPs	Gold Nanoparticles
LB	Lysogeny Broth
TEM	Transmission Electron Microscopy
FPLC	Fast Protein Liquid Chromatography

List of Chemicals

$AuCl_4$	Chloroauric Acid
$NaBH_4$	Sodiumborohydride
$NaCNBH_3$	Cyanoborohydride
NaCl	Sodium Chloride
TRIS	2-Amino-2-hydroxymthyl-propane-1,3-diol
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
NaH_2PO_4	Sodium phosphate monobasic

1. Introduction

1.1 King's college London (BPO laboratory)

The BPO lab, has used protein nanocages (i.e. ferritin) to control the generation of inorganic nanomaterials and also in the engineering, manipulation and fundamental biophysics of protein cages. Their laboratory research work explores the folding and assembly of ferritin proteins, by engineering them to express unique functions which can also be used as nano reactors for gold and silver nanoparticles. Finally, the lab explores the development of supra-assembled ferritin arrays that could contain nanoparticles of various sizes but further work would be need to realise this potential.

1.2 Background and project presentation

In recent years, metallic nanoparticles have gained extensive relevance, their multiple applications and diverse properties have attracted extensive attention.

Currently, nanoparticles are making significant contributions in many areas such as medical diagnosis, therapeutics, sensors and electronics. [1] With such wide ranging applications, many methods have been discovered to help in the synthesis of nanoparticles.

Many of these methods use chemicals that require very specific conditions such as organic solvents, high temperatures and pressures and strong reducing agents. [1]

Recently, a new method to fabricate nanoparticles has been achieved. This method uses protein shells as templates in their fabrication. Using biological sourced protein shells was required an intensive research to find what methods could be used to grow nanoparticles inside, as often they have stability concerned. Therefore, processes that avoid extreme conditions like some of the previous examples would be preferred.

During this work, a biotemplated method to produce gold nanoparticles will be expanded.

The objective of this project is to expand a method which allows for the fabrication of gold nanoparticles inside of highly stable mammalian unmodified protein nanocages, to less stable bacterial nanocages which exhibit other symmetries such as Bacterioferritin (BFR) and DNA-binding protein from starved cells (DPS).

1.3 Contributions of the work

This research contributes to the investigation of the production of gold nanoparticles inside BFR and DPS with the objective to describe what method is more suitable for the production of the gold nanoparticles.

In this project was necessary to deepen concepts and knowledge in FPLC previous developed in the research group and also extend a new method to the fabrication of the gold nanoparticles inside the two different ferritins.

1.4 Thesis organisation

This thesis is organised into five main chapters:

- **Chapter 1** states the objectives of this project as well as explaining previous work done in this field.
- **Chapter 2** is an introduction to nanoparticles, gold nanoparticles and ferritin proteins including their advantages and applications.
- **Chapter 3** presents all of the experimental work procedures including equipment, materials and methods used.
- **Chapter 4** presents the results and discussion of the experimental work.
- **Chapter 5** presents the conclusions that summarize the results of the experimental work and future work.

2. State of art

2.1 Gold nanoparticles

The field of nanotechnology has attracted the attention of the scientific community in recent years. [1-2]

Nanoparticles (NPs), with their specific properties such as physical, chemical and biological properties (lower melting points, specific surface areas, mechanical strengths and specific optical properties) are in the middle of an innovation revolution with a promising future ahead.[1-2] NPs are complex compounds with a dimension between 1 and 100nm showing specific properties that make them different from other particles with bigger dimensions. In fact, the surface area of these particles compared to bigger particles appears to be one of the key reasons for their unique properties. Currently, nanoparticles are useful in a large and diverse range of applications in multiple areas include: mechanical (improve the resistance of mechanical devises and also give anti-corrosion abilities), energy (could improve solar panel efficiency and produce efficient fuel cells), optical (NPs could be used for anti-reflection product coatings), biomedical (have been used to produce “quantum dots,” to detect diseases, cancer treatments and could also be used to change the flavor and color of food and drinks).[3]

Gold nanoparticles (AuNPs) have interesting properties such as their electronic, magnetic and optical properties [4-5] and also their usefulness in a variety of applications such as in catalysis, optical sensors and in medical diagnosis.[1]

The size and shape of the gold nanoparticles effects their solubility, reactivity and photonic properties. It is therefore essential to develop efficient methods to control the size and shape of these nanoparticles.[6]

Crystallinity and surface facets are the two major structural factors that control gold nanoparticle shape. While crystallinity is defined in particle formation in the initial nucleation stages, the surface facets is defined during the growth of the nanoparticle. [2]

2.2 Ferritins

Ferritin proteins were first described by Laufberger in 1937 and still remain at the cusp of research today showing new and unexpected characteristics. Ferritins are known by their three-dimensional structure capable to store large amounts of iron in a soluble and disposable form.

The ferritin family is wide and diverse and is found in both eukaryotes and prokaryotes. The ferritins present in eukaryotes are hetero-oligomers and contain both a structural and a catalytic monomer which is used for iron mineralisation. On the other hand, prokaryote ferritins are homo-oligomers and contain a single monomer needed for both the structural and catalytic functions for iron mineralisation and storage. [7]

Ferritin proteins have the capability to form hollow cages. These proteins are present in nature and are involved in iron detoxification and storage, storing iron as a mineral in the hollow cavity.[8] Ferritins have been used to synthesize various types of nanoparticles such as metals, metal oxides and semiconductors.[9] Mammalian ferritins, for example HsFn are composed of two different subunits, the heavy chain (H) composed by 178 amino acids and the light chain (L) with 174 amino acids. The H-chain is responsible for the ferrioxidase activity and the L-chain stabilizes the nanocage structure.[10] In addition, the hydrogen bonds, salt-bridges and hydrophobic interactions are responsible for the ferritin folding and stability. The stability of the H- and L-subunits differ significantly. Hydrogen bonds are more abundant in the H-subunit (about 50% more) compared with the L-subunit, however salt bridges are much more abundant in L subunit that highly contributing for its higher stability.[10]

Ferritins store iron as ferrihydrite, a semi-conductor mineral with the capacity to catalyze oxidation/reduction reactions. The ferrihydrite mineral is stored inside the cavity of the ferritin proteins, without any disruption to the quartenary structure at either high pH (from pH 2 to 10) or temperature (up to 70ºC). These properties help in the removal of the mineral core allowing the empty cages to be used (apoferritin).[11]

Horse spleen ferritin

Horse spleen ferritin (HSfn) is a semispherical ferritin found in horses and has an exterior diameter of 12nm and a cavity diameter of 7nm. HSFn is a ferritin from the spleen of the horse and due to is singular structure and stability is a great model to fabricate nanoparticles with an homegeneous shape and size.[10] HSFn can be used to store molecules in its interior cavity that fact makes the protein a good template to produce different nanoparticles with different properties.[10]

Bacterioferritin

Bacterioferritin is a ferritin isolated from the *E.coli* bacterium. BFR from *E.coli* has the ability to form a homo-meric nanocage with octahedral symmetry. This ferritin is known as "maxi ferritin" composed of twenty-four subunits assembling along two-, three- and four-fold axes of symmetry. BFR has an exterior diameter of 12nm and an interior cavity diameter of 7.5nm.[11]

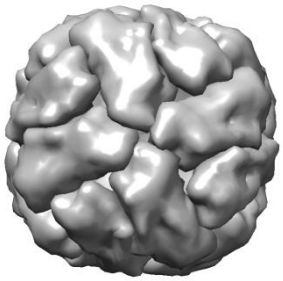

Figure 1- Bacterioferritin (BFR)

DNA binding protein from starved cells (DPS)

DNA-binding protein from starved cells (DPS) is known as a "mini ferritin" composed of twelve subunits. DPS are induced in microorganisms by nutritional stress and have a principal function of protecting DNA from damage. DPS has an exterior diameter of 9nm and an interior cavity diameter of 4.5nm.

The hydrophobic interactions that occur in this protein lead to the formation of a 4 helix-bundle in each DPS subunit. [13]

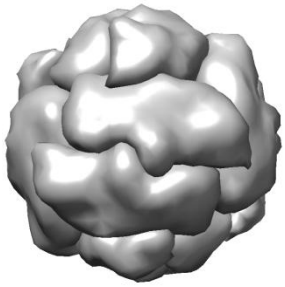

Figure 2- DNA-binding protein from
starved Cells (DPS)

BFR and DPS

Even though the amino-acid sequence in BFR and DPS is very different these two proteins have very similar tertiary structure. However, the quaternary structures of these ferritins are very different. The major difference is that BFR contains an extra helix at the C-terminus. As opposed to BFR, instead of an E-helix DPS has an additional helix, known as "BC-helix on a loop between the second and third helices.

Nanoparticles and the ferritin family

Nanoparticles grown inside ferritins have a confined distribution size due to the ferritin cavity size. They are able to increase the chemical and solubility stability of the particles and having protein shells allows for larger applications through protein engineering. The potential for so many useful applications has encouraged research into methods to grown nanoparticles inside of ferritin cages. However, initial designs could only be achieved by first modifying the protein to enhance the ion binding affinity and therefore localization of gold inside of the nanocage to promote the formation of nanoparticles. In order to facilitate the synthesis of different types of nanoparticles such as carbonates semiconductors and metals. Nanocage proteins have well defined size and shape, which results in encapsulated nanoparticles being surrounded by a soluble protein shell in a biological media, and with better protection to the particles from aggregation.

The method that is used in this project describes a technique to grow gold nanoparticles inside three different ferritins without the need to modify

the protein. Hopefully this will expand this method enough so that it could be used for any nanocage or capsid protein.

2.3 Characterization techniques for the production of gold nanoparticles

In order to produce the gold nanoparticles several techniques were used. In this chapter, these techniques will be summarized.

Fast protein chromatography (FPLC)

Fast protein liquid chromatography (FPLC) is a liquid chromatography method used to analyse, identify and purify proteins. FPLC uses columns that are composed for two different materials, the stationary phase and the "mobile phase". The mobile phase passes through the column (stationary phase).The mobile phase is normally a buffer. Buffers are commonly used in FPLC rather than organic solvents and have the advantage that their pH changes very little when an acid or base is added. Buffer solutions are used to maintain the solution pH, which should increase protein survivability during purification or analysis processes.[12]

The FPLC machine is composed of:

- one, two or more pumps that have the function to pump the buffer into the column;
- an injection loop: a segment of tubing used to inject the sample inside the column. The sample normally is injected by a syringe or in a superloop (if the sample is bigger than 2mL);
- an injection valve: links the mixer and the sample loop to the column;
- column: could be plastic or glass cylinder packed with beads of resin, normally agarose;
- flow cell: the sample passes through a flow cells to measure the concentration of protein in the effluent (UV light absorbance at 280nm);
- monitor: The flow cells are connected to a monitor. The monitor permits visualisation of the protein as it elutes;

- collector: is a rotating rack that could be filled with test tubes to collect the sample.

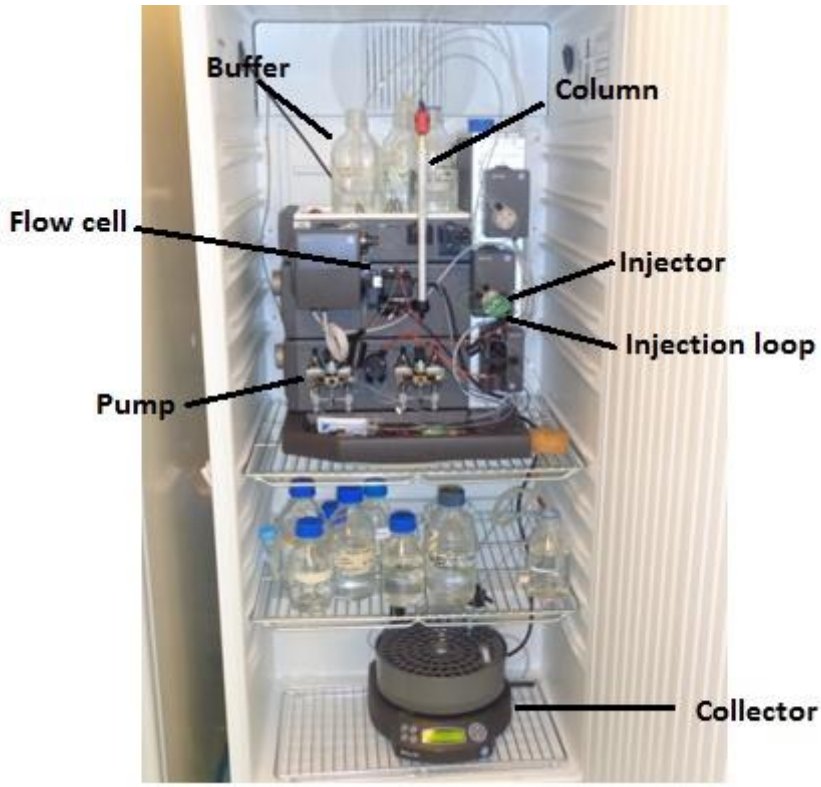

Figure 3- Fast protein liquid chromatography (FPLC)

Histrap

Histrap is a fast protein liquid chromatography (FPLC) strategy used to collect the 6x-histagged proteins present in the sample. During a histrap the protein of interest will bind in the histrap column. Two buffers are used during a histrap, the first is a binding buffer which allows the 6x-histagged protein to bind to the matrix due to its low imidazole concentration. The second, which contains a much higher concentration of imidazole, competes for the matrix and 'knocks off' the 6xhis tagged protein. The effluent passes into a detector that measures the protein concentration which allows to select which fraction to collect.

Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) is another FPLC method. In SEC, molecules are separated by their size. A mixture of solutes of different sizes passes into a porous column. The large molecules do not penetrate the pores packed in the column and therefore have a short path length and elute first. The smaller molecules can penetrate into the pores and elute later.

Transmission electron microscope (TEM)

Transmission Electron Microscopy (TEM) operates with the same basic principle as the light microscope however instead of using light it uses electrons. In TEM a beam of electrons is transmitted through a vacuum microscope column. This uses an electromagnetic lens to focus the electrons into a beam. The electron beam travels to the sample. In the bottom the unscattered electrons hit the fluorescence screen giving a shadow image of the sample. [13]

3. Experimental work

The main goal of this project is to expand the production of gold nanoparticles inside unmodified protein cages: BFR and DPS using the fabrication of gold nanoparticles inside HSFn as a control. For that, many methods were attempted in order to discover what was the best technique for the production of the gold nanoparticles.

A previously published method to grow gold nanoparticles inside of unmodified HSFn by the Orner lab, was the starting point for this investigation. This method starts by doping an apoferritin (empty cavity) solution with a chloroauric gold solution ($AuCl_4$). The intension being that the gold ions can diffuse into the empty cages and form a concentration equilibrium between the solution and the protein cavity. After three hours of incubation, the protein solution is desalted, this separates the protein cage and any gold that is incorporated into it, from any gold ions left in the solution. The next step is to add a strong reducing agent ($NaBH_4$) to reduce any trapped gold into a small nanocluster which should be too big to diffuse out of the protein nanocage pores. The final stage is to add additional gold ions and a weaker reducing agent that should only be able to reduce gold ions in the presence of an already reduced species (nanocluster), this expands the nanocluster into a nanoparticle the same shape and size as the nanocage.[14]

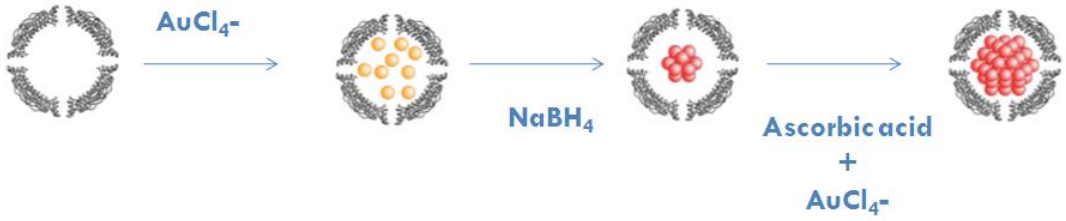

Figure 4- Schematic representation of the two step procedure to use HSFn to mineralize gold nanoparticles from a previously reported method

3.1 Description of the experimental work

Protein production

The experimental work began with the production of BFR and DPS. Below are the experimental steps necessary to express and extract the protein. Firstly it is necessary to make a nutritionally rich medium, Lysogeny broth (LB) and a pre culture with the bacteria that wants to grow.

1. **LB**

To make 1000mL of LB is necessary 10g of Sodium chloride (NaCl), 10g of tryptone and 5g of Yeast extract.

The sodium chloride has as function the transport and the osmotic balance provided by sodium ions.

The tryptone is used to provide peptides, peptones and the aminoacids to the growing bacteria.

The yeast extract is used to give to the bacteria the nutrients necessary to grow.

2. **Pre culture**

The experimental work began with the production of BFR and DPS. Below are the experimental steps necessary to express and extract the protein. Firstly it is necessary to make a nutritionally rich medium, Lysogeny broth (LB) and a pre culture with the bacteria that wants to grow which contains a plasmid with the gene for the protein of interest.

The pre culture volume of 1% of the total LB volume should be used. To 10 ml of LB, 10uL of antibiotics (carbenicillin) and 10uL of DPS or BFR in stock is added. The carbenicillin is a bacterial antibiotic and is used to prevent any type of contamination.

The pre culture grows over night at 37 ^{0}C. In the next day carbenicillin was added to the autoclaved LB (1uL of carbenicillin for 1 ml of LB) and the pre culture. The mixture was incubated at 37ºC for 3-4hours. After 4 hours the optical density (OD) was tested in an absorbance at 600nm and when the OD was in the range 0.5-0.6 IPTG (isopropyl ß-D-1-thiogalactopyranoside), (0.5uL of IPTG for 1mL of LB) was added. The IPTG is a reagent used to stop bacteria to grow to start focusing energy on expression the protein.

After that the mixture was incubated at 30º C for 3hours while shaken.

After 3 hours the culture was centrifuged into a pellet and placed at 80^{o}C freezed overnight.

The next day, the pellet was defrosted and 15mLs of lysis buffer was added.

The lysis buffer (50mM NaH_2PO_4, 300mMNaCl and 10mM Imidazol) has an alkaline pH and contains NaCl to promote the concentration and retention of the protein.

The sample was sonicated (cycle of 10s pulses on/off at 15% amplitude for 10 minutes).

The sonication process is used to lyse the cells releasing them into the buffer. This process was performed in an ice bath to avoid protein denaturation due to the heat occur during the application of the method.

After sonication, the sample was centrifuged at 30,000rpm for 20min at 4º C to separate the insoluble and unlysed cells from the soluble proteins that have been released.

Histrap

DPS as demonstrated in figure 5 is composed of a monomer and a histag. The histrap was performed on a GE Akta FPLC system using an Histrap

HP 5ml column at a flow rate of 2mL/min with two running buffers: the binding/washing buffer (50mMNaH_2PO_4, 300mMNaCl and 20mM Imidazole) and the elution buffer (50mM NaH_2PO_4, 300mMNaCl and 500mM Imidazole).

This method is used to remove the histaged protein present in the sample from all other proteins in the lysate. As DPS is expressed with only a short tag, the protein cage can assembly without any digestion steps required. Size exclusion chromatography is used as an orthogonal purification method.

Contrariwise BFR as shown in figure 6 is expressed as a complex containing the monomer, a histag and also a trxtag. After BFR has been semi purified via histrap is not able to form a cage because it still has large sterically clashing tags attached. Another step is needed, which uses the enterokinase enzyme to digest the tags off. This is performed in enterokinase buffer (20mM Tris,50mM NaCl and 2mM $CaCl_2$) and enterokinase enzyme (10 ul), 2 days at 4 ^{0}C. After that the size exclusion chromatography (SEC) is performed.

DPS

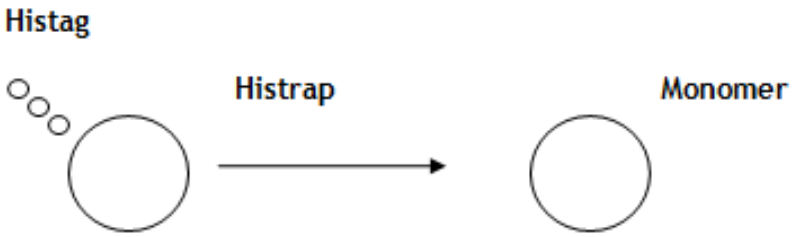

Figure 5- Schematic representation of DPS

BFR

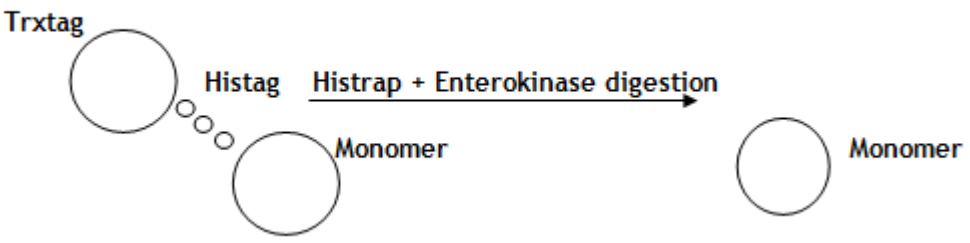

Figure 6- Schematic representation of BFR

Size exclusion chromatography (SEC)

The size exclusion chromatography was performed on a GE Akta FPLC system using a Hiload 16/600 Superdex 200pg gel filtration column at a flow rate of 1ml/min with a running buffer composed by 50mM NaCl and 50 mMtris, pH 7.8.

Protein quantification (BCA method)

The Bicinchoninic acid (BCA) is a colorimetric method that allows the detection and the quantification of the protein. The chelation of two BCA molecules with a Cu ion (formed by the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium) forms a purple color complex which exhibits a strong absorbance at 562nm that increase with the protein concentration. The complex color formation depends on the macromolecular structure of the protein, on the number of peptide bonds and the presence of some amino acids in particular cysteine, tryptophan and tyrosine. [15]

Production of gold nanoparticles inside horse spleen ferritin (control method)

Protein purification

Horse spleen ferritin (HSFn) was purchased (Sigma) and stored at -20 ^{0}C in a glycerol solution. The stock solution was subjected to SEC performed on a GE Akta FPLC system using a Superdex 200 10/300 GL, gel filtration column at a flow rate of 0.5ml/min column equilibrated with a buffer composed with 50mM NaCl and 50mM TRIS with a pH of 7.8.The fractions were collected and the concentration checked by BCA method.

Production of the gold nanoparticles (general method)

To the purified protein, 22.6 ul of 0.1 M chlorauric acid ($HAuCl_4$) solution was added into 1ml of purified HSFn solution (1mg/ml solution) with

a molar ratio of 1000:1. The mixture was incubated for 3hours at room temperature.

After 3 hours the mixture was applied to a desalting column (Sephadex G-25,GE Healthcare) at a flow rate of 3ml/min equilibrated with a buffer composed of 50mM TRIS with a pH of 7.5. The desalting process was applied to remove an unencapsulated gold from the sample.

After this was desalted the fractions which contain the protein were combined and 20 ul of a 0.1 M solution of a strong reducing agent, sodium borohydride ($NaBH_4$) was added. This first reducing step is applied to form small nanoclusters inside the ferritin. The mixture was incubated for 3hours at room temperature.

After 3hours, 30ul of a 0.1M solution of a weak reducing agent, ascorbic acid and an addition 10 ul of 0.1 M $HAuCl_4$ were added. This second reducing step is applied to expand the nanocluster into a nanoparticle and fill up the protein cavity. The mixture was incubated overnight with no agitation at room temperature. The day after, the supernatant was subjected to a SEC performed on a GE Akta FPLC system using a Superdex 200 10/300 GL, gel filtration column equilibrated with a buffer (50mM NaCl and 50mM TRIS, ph 7.8).

4. Results and discussion

In this chapter will be presented the results obtained for the production of gold nanoparticles inside HSFn (control method), DPS and BFR.

4.1 Quantification of the protein (BCA method)

The ferritin was quantified with the BCA method described in chapter 3. The calibration line was constructed using pre made BSA standards which allow for the concentration of the ferritin sample to be calculated.

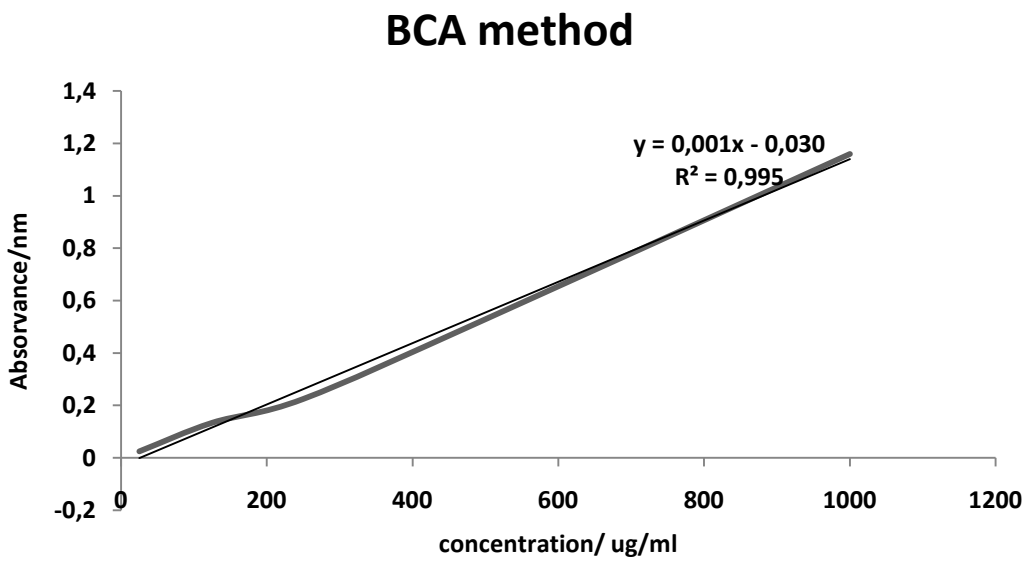

Figure 7- Calibration line obtained for BCA method

4.2 Production of gold nanoparticles inside Horse spleen ferritin (control method)

The main method of analysis for nanoparticle experiments is SEC. This method allows reproducible identity of the elution volume associated with each of the proteins that are being tested. Samples that provide peaks at these elution volumes are good indicators that the experiment allowed the protein to remain intact, with the possibility of nanoparticles been localized inside of them. For HSFn the elution volume for the cage protein is 12 mls.

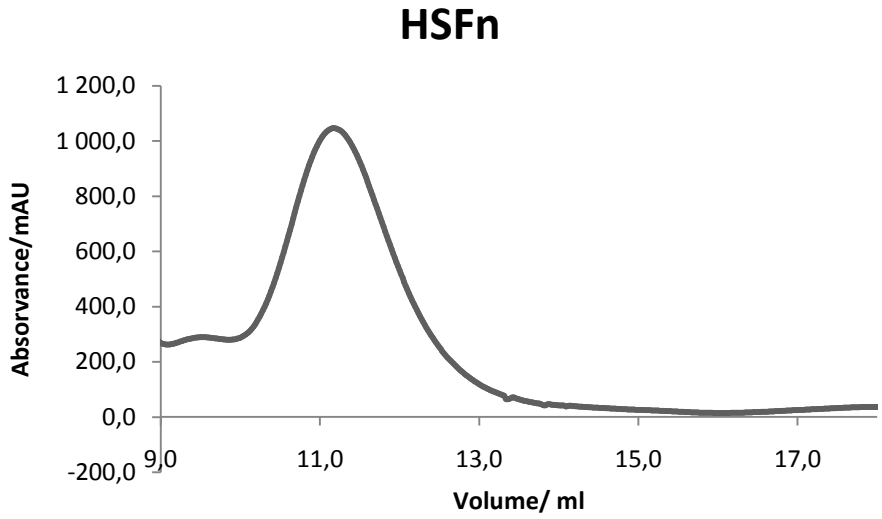

Figure 8- SEC for HSFn

The SEC results for the method described above are shown in figure 9.

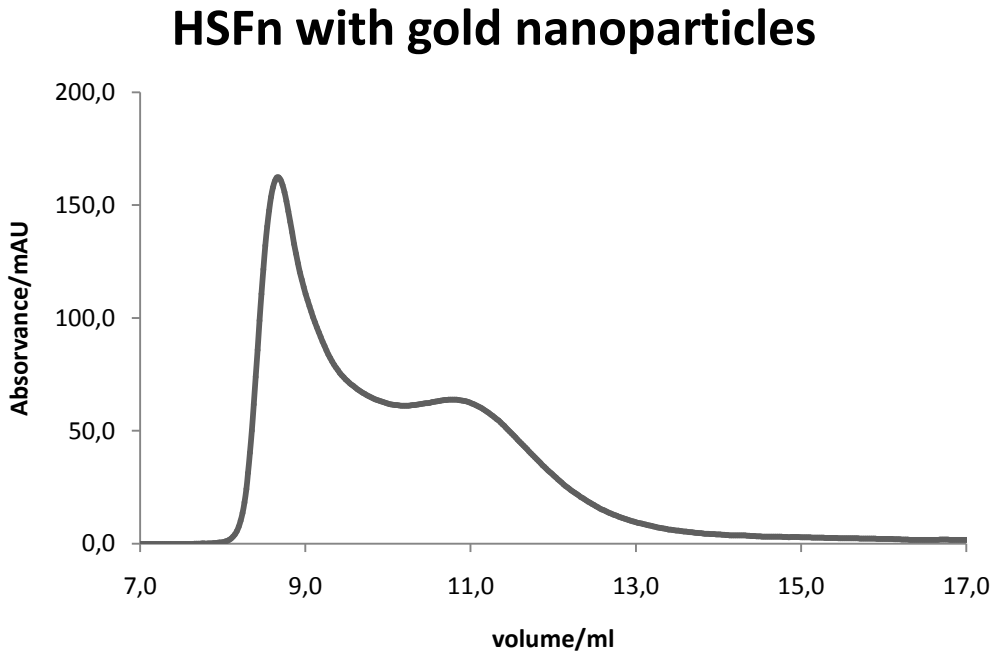

Figure 9- SEC for HSFn with gold nanoparticles integrated

It is possible to confirm that the gold nanoparticles are integrated inside HSFn. The NPs has the same elution peak as HSFn.

Figure 10 shows a red sample collected after the SEC that proves the gold nanoparticles are present.

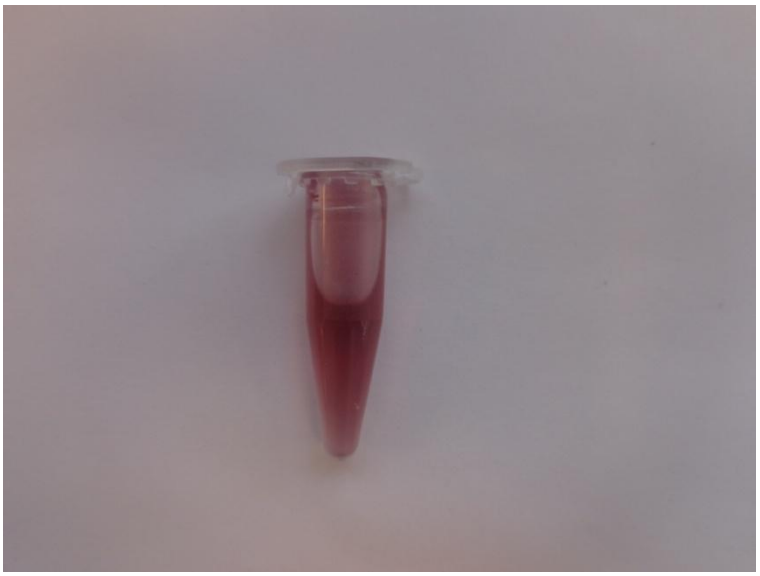

Figure 10- Red sample collected after the SEC

Figure 11 shows TEM images obtained from gold nanoparticles inside HSFn.

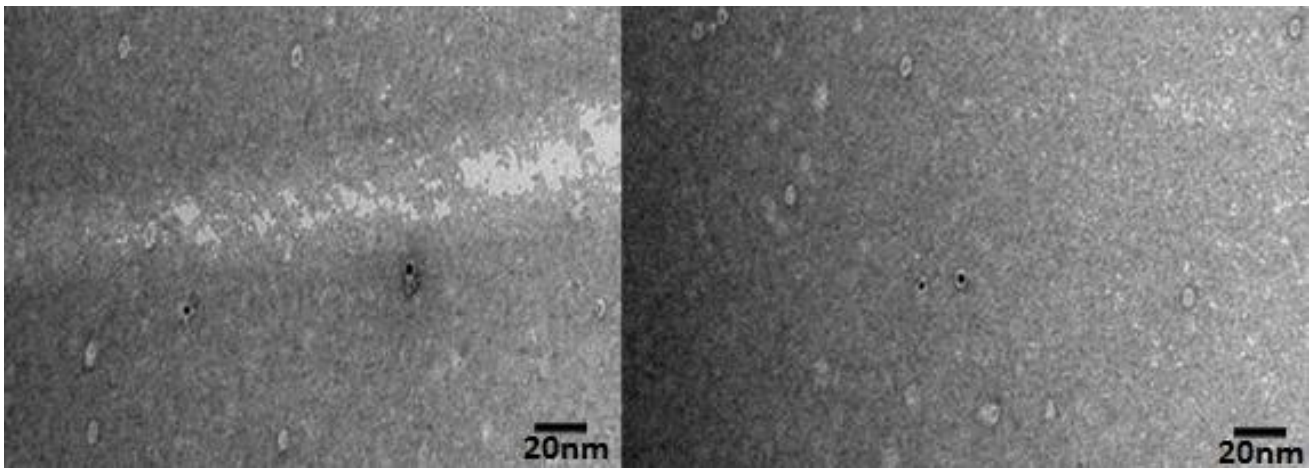

Figure 11- TEM image AuNPS in HSFn; TEM: HV: 120kV

TEM provides additional evidence for the present of nanoparticles inside of ferritin cages. The ideal image is one that shows both a dark dot (nanoparticle), with a white halo around it (ferritin cage). This should confirm encapsulation. However, is also wish to see the presence of empty cages,

white rings with no dot, as this provides evidence for the survival of the ferritin nanocage quaternary structure. The nanoparticles obtained here are mono dispersive and spherical. The average diameter is 6nm which match to the dimension found in literature (7nm).

4.3 Production of gold nanoparticles inside DPS

The elution volume of DPS was observed at 12 ml on a SEC column, as seen in figure 12.

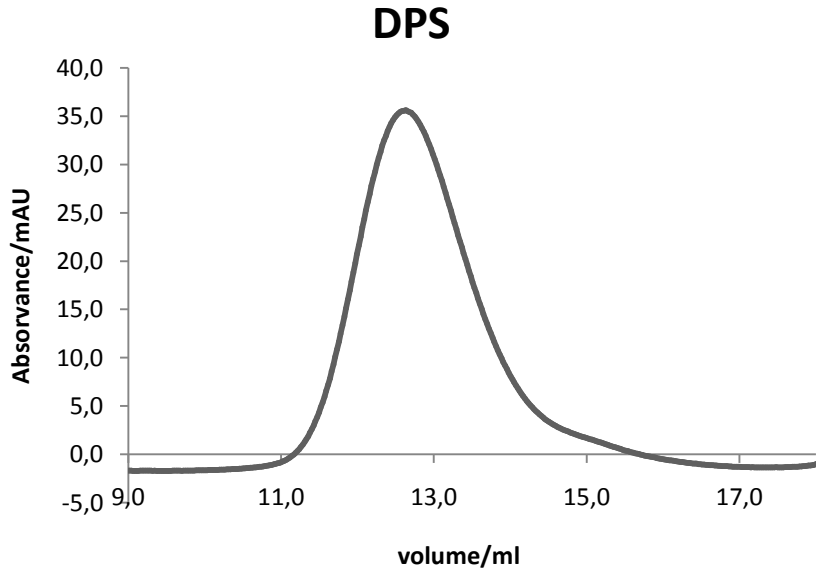

Figure 12- Size exclusion chromatography of DPS

The method described to produce gold nanoparticles inside ferritins was applied in DPS the image 13 shows the SEC obtained.

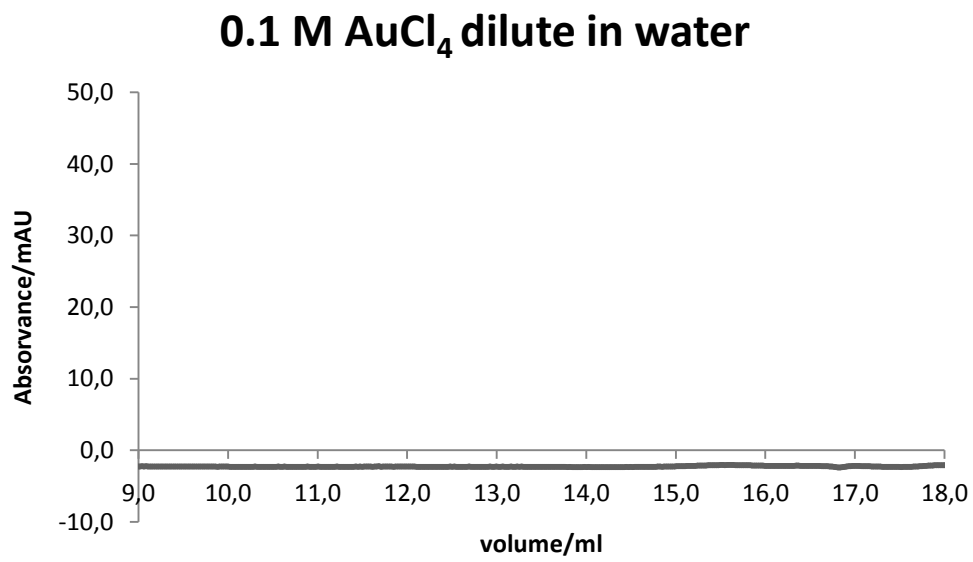

Figure 13- SEC of DPS in 0.1M AuCl4 dilute in water with AuNPs integrated

There is no detectable cage peak maybe because DPS lose its quaternary structure, preventing the formation of encapsulated nanoparticles.

Survability test for incubation with gold, DPS

The original method to produce gold nanoparticles inside ferritins was unsuccessful. A new approach was taken, which involved deconstructing this method into its component parts. Each part was then modified and the protein quaternary structure was analyzed to examine whether a better method could be found to work with DPS. It is hoped that by examining each part and ensure protein survivability at every level, we can find a new method that will produce intact ferritins with nanoparticles when each individual step is recombined.

Step one, which is the incubation of gold ions with the protein cage, was rested but under different conditions. The concentration, buffer, pH and volume added were analyzed

In table 1 is shown the results obtained for these test.

Table 1- Survability test of DPS in $AuCl_4$

0.01 M $AuCl_4$ in water	DPS cage peak observed
0.01 M $AuCl_4$ in water added slowly over 30min	DPS cage peak observed
0.1 M $AuCl_4$ in TRIS	DPS cage peak observed
0.01 M $AuCl_4$ in TRIS	DPS cage peak observed
0.01 M $AuCl_4$ in TRIS added slowly over 30min	DPS cage peak observed

The elution volumes of the experiments were obtained by SEC and are showing in figure 14, 15, 16, 17 and 18 respectively.

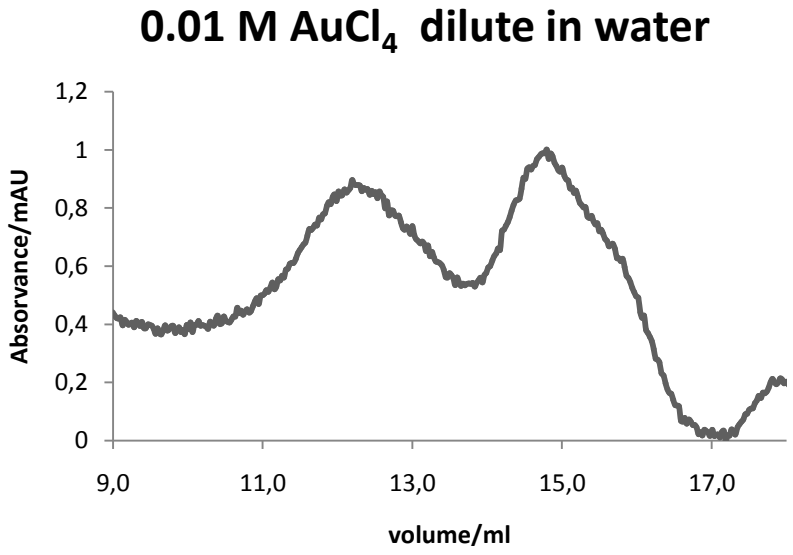

Figure 14- SEC of DPS in 0.01M AuCl4 dilute in water

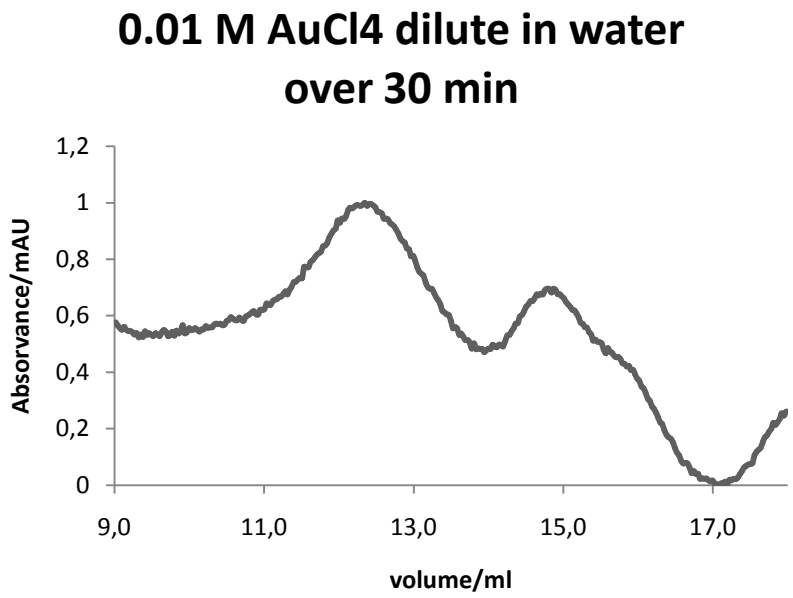

Figure 15- SEC of DPS in 0.01 M AuCl4 dilute in water over 30min

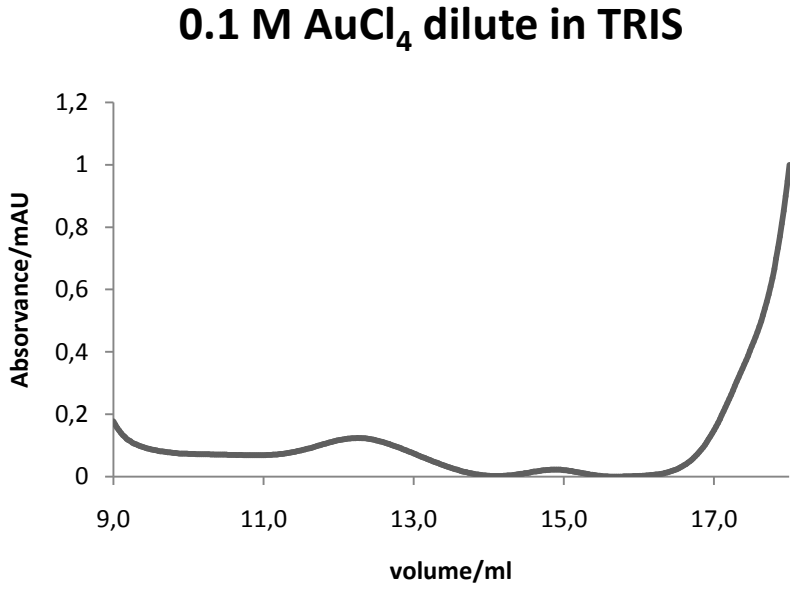

Figure 16- SEC of DPS in 0.1 M $AuCl_4$ dilute in TRIS

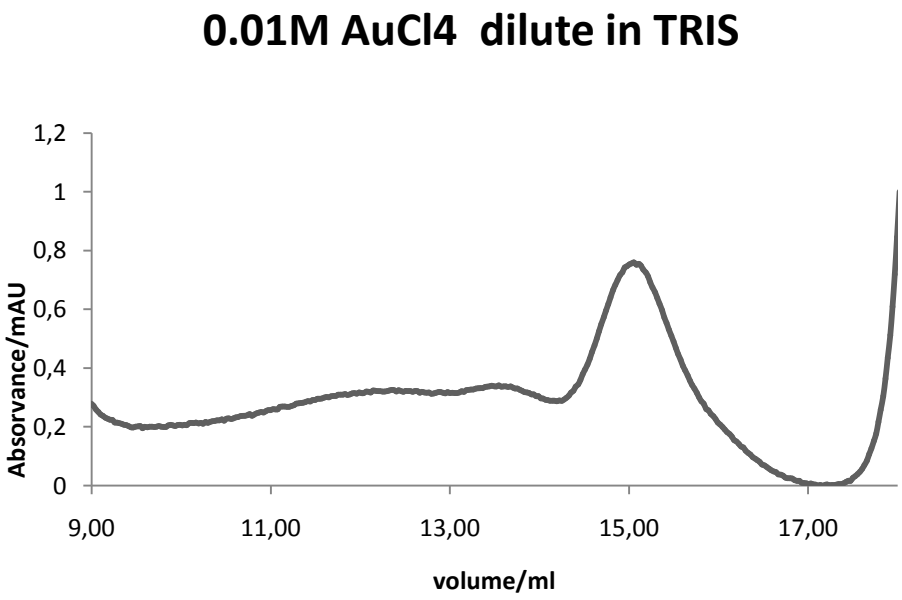

Figure 17- SEC of DPS in 0.01 M $AuCl_4$ dilute in TRIS

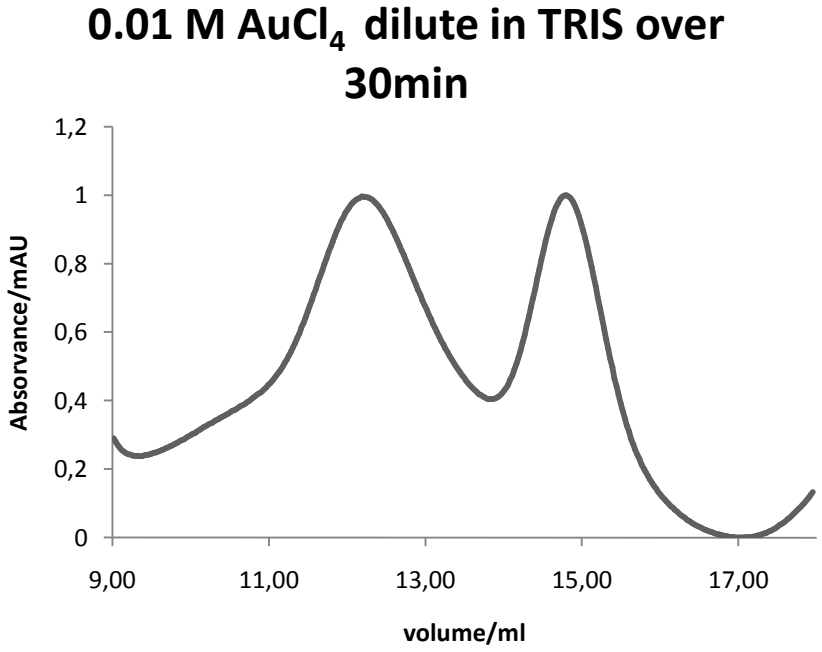

Figure 18- SEC of DPS in 0.01M $Aucl_4$ dilute in TRIS over 30 min

A cage elution peak at 12 ml is observed for all of the above experiments. A second peak at 15 mls is also seen, it is believed that this is a dimer intermediate which is often seen in ferritin samples. While none of the experiments match the positive control DPS sample, there is at least some protein cage survivability.

Survivability test for first reduction of gold, DPS (2ND STEP)

After testing the survability of DPS in the presence of $AuCl_4$ the next step was producing the gold nanoclusters inside of the ferritin. This was done by the addition of 20 ul 0.1 M $NaBH_4$ after the initial gold incubation step. This was then left for 3 hours at room temperature before being analyzed via SEC.

In table 2 is shown the results obtained for these experiments.

Table 2- Results for the production of gold nanoparticles inside DPS

0.1 M $AuCl_4$dilute in water	No detectable protein cage
0.01 M $AuCl_4$dilute in water	No detectable protein cage
0.01 M $AuCl_4$dilute in water over 30min	No detectable protein cage
0.01 M $AuCl_4$dilute in TRIS	Detectable protein cage
0.01 M $AuCl_4$dilute in TRIS over 30min	Detectable protein cage

The elution volumes of the experiments were obtained by SEC and are shown in figure 19, 20, 21 and 22 respectively.

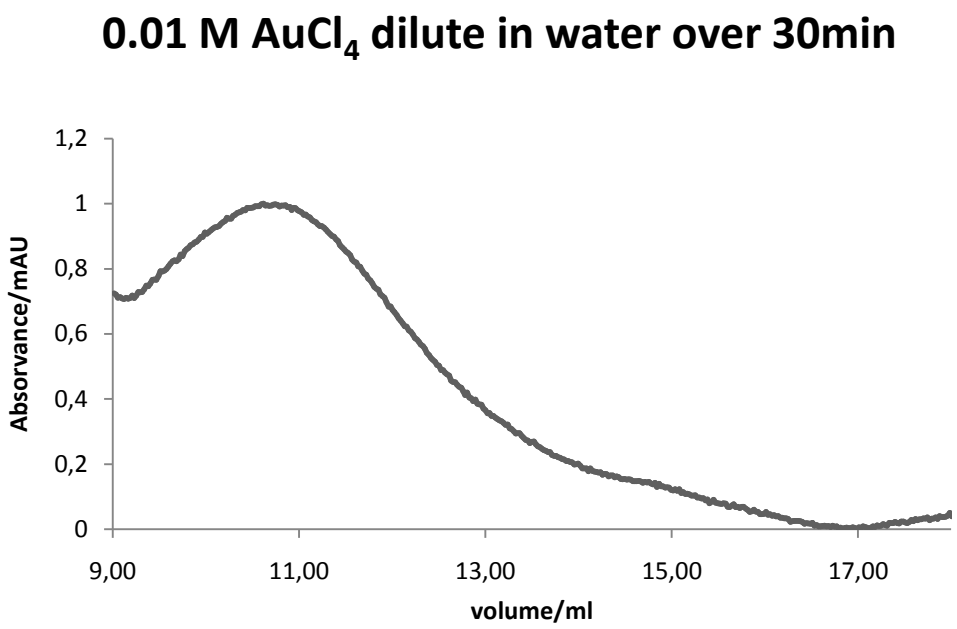

Figure 19- SEC of DPS in 0.01M AuCl4 dilute in water with AuNPs integrated

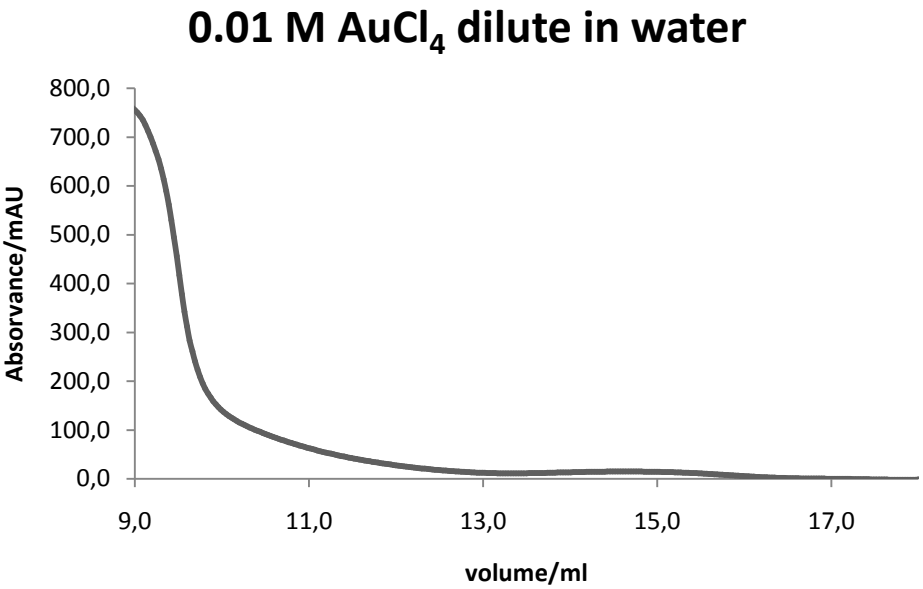

Figure 20- SEC of DPS in 0.01M $AuCl_4$ dilute in water 0ver 30min with AuNPs integrated

In figure 19 and 20 is possible to observe that there are no peaks at 12 ml, elution volume that characterizes DPS which is conclude that those two experiments did not work.

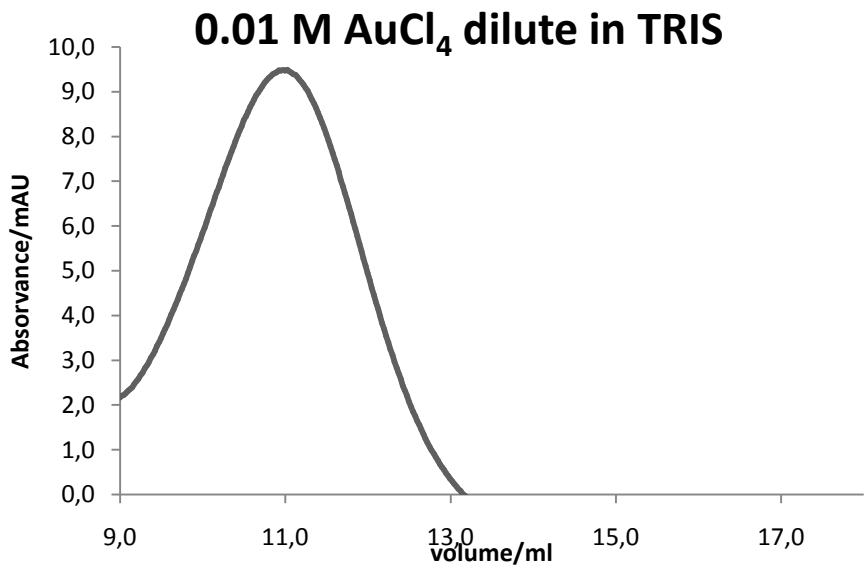

Figure 21- SEC of DPS in 0.01M $AuCl_4$ dilute in TRIS with AuNPs integrated

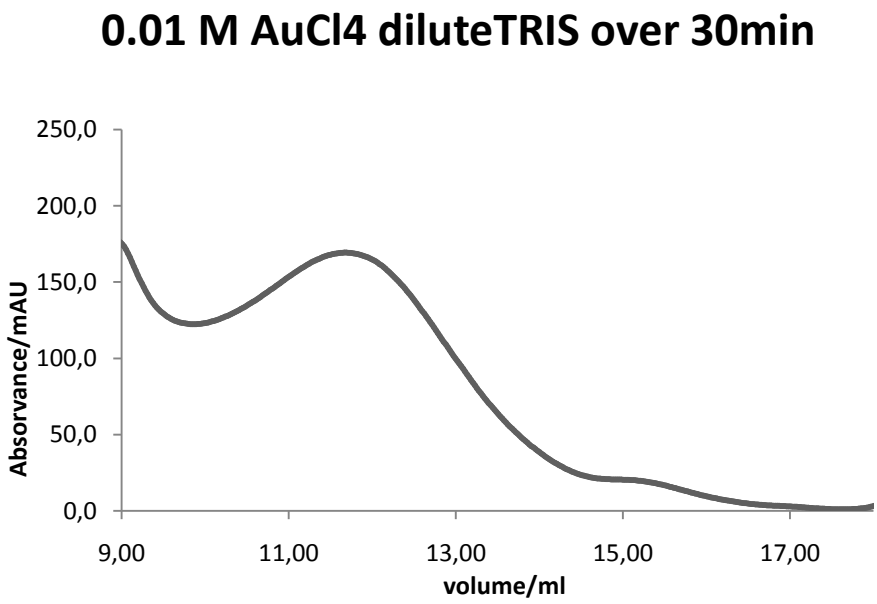

Figure 22- SEC of DPS in 0.01M AuCl4 dilute in water TRIS with AuNPs integrated

It is possible to observe in figure 21 and 22 that there are peaks at 12ml. In conclusion observing these images it is suggested that the protein

survived and the experiment work.

After analyzing the second step, is possible to see that there are some methods that still show a protein cage peak at 12 mls after SEC. This is encouraging news and allowed to examine the third and final step with confidence. The third step was tested the ascorbic acid a reagent used as reducing agent. The SEC image is shown in figure 23.

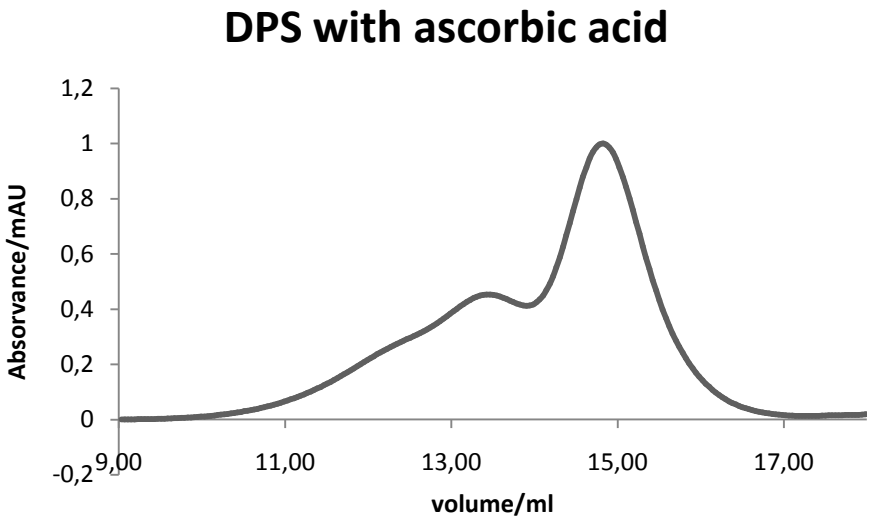

Figure 23- SEC of DPS in the presence of ascorbic acid

It is possible to observe in figure 23 that is not a certain peak at 12ml. In conclusion observing this image it is suggested that the ascorbic acid seemed to be playing a role in protein denaturation.

The next step was to see the effect of different reducing agents on DPS protein cage survivability. To that end, a new method was preformed as described. To DPS 20uL of chloroauric acid ($HAuCl_4$) 0.01M solution which was dissolved in TRIS-NaCl buffer (50mM TRIS, 50mMNaCl,pH 7.8) was added five times with ten minute gaps in between. The mixture was incubated for 3hours at room temperature. After 3 hours the mixture was applied to a desalting column (SephadexG-25, GE Healthcare) at a flow rate of 3ml/min equilibrated with a buffer composed with 50mM TRIS and 50mM NaCl with a pH of 7.8.

After desalting the fractions which contain protein were combined and 100 ul of 0.1M cyanoborohydride ($NaCNBH_3$) diluted in TRIS-NaCl buffer was

added. The mixture was incubated for 3hours at room temperature.

After 3hours 40uL of a 1M solution of trisodium citrate and more $HAuCl_4$ was added (20ul, 0.1M) were added. The mixture was incubated overnight with no agitation at room temperature. The day after, the sample was subjected to a SEC performed on a GE Akta FPLC system using a Superdex 200 10/300 GL, gel filtration column equilibrated with buffer(50mMNaCl and 50mM TRIS, pH 7.8) as shown below.

In figure 24 is possible to see the elution volumes obtained by SEC.

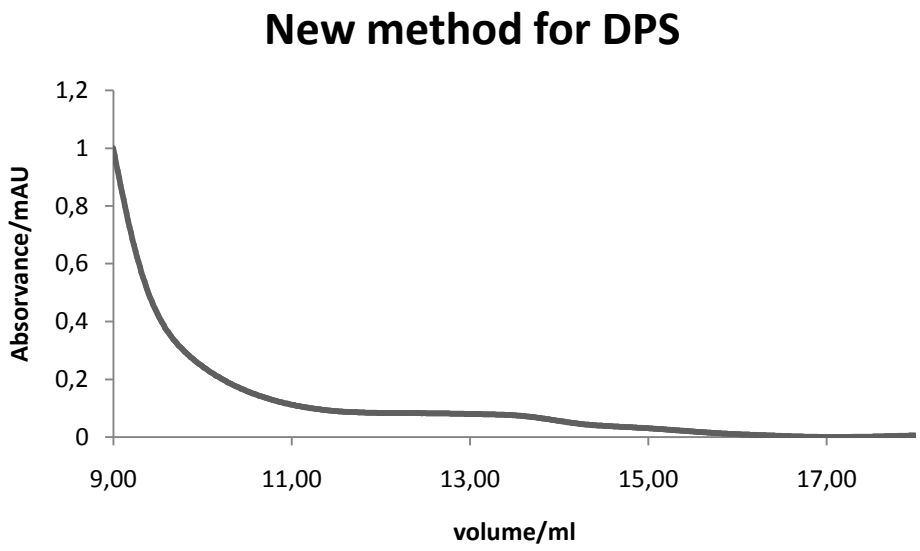

Figure 24- SEC of DPS in the new method with NPs integrated

While a small cage peak was observed showing for the first time the complete protein cage after the completion of the whole process, no nanoparticles were detected as observed by the color of the sample.

4.4 Production of gold nanoparticles inside BFR

The elution volume of BFR as observed from SEC is 11 mls for the cage peak. Interestly, for BFR we also observe a dimer peak at 15 mls as shown in figure 25.

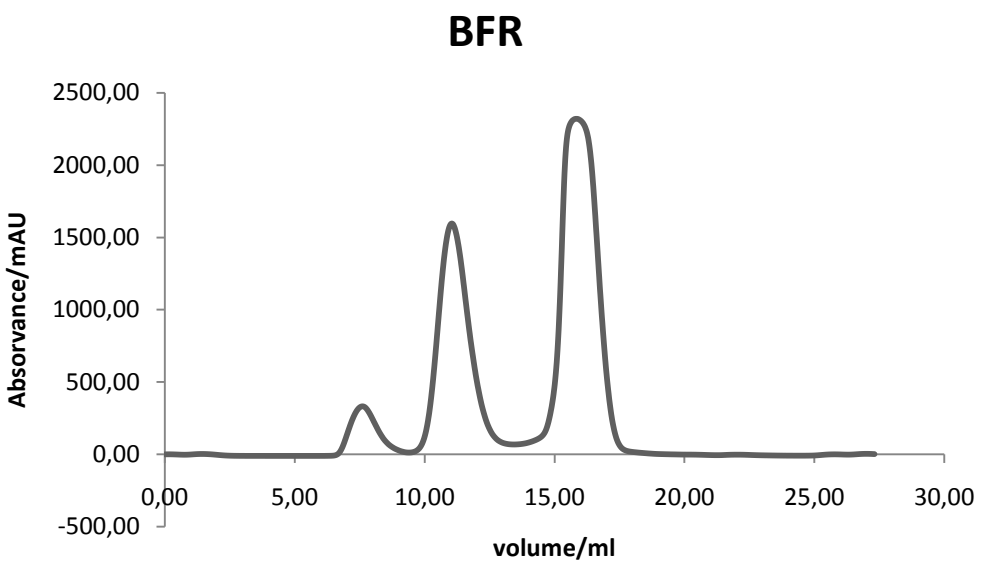

Figure 25- Size exclusion chromatography of BFR

The original method to produce nanoparticles inside of maxi ferritins as described was applied to BFR as shown in figure 26.

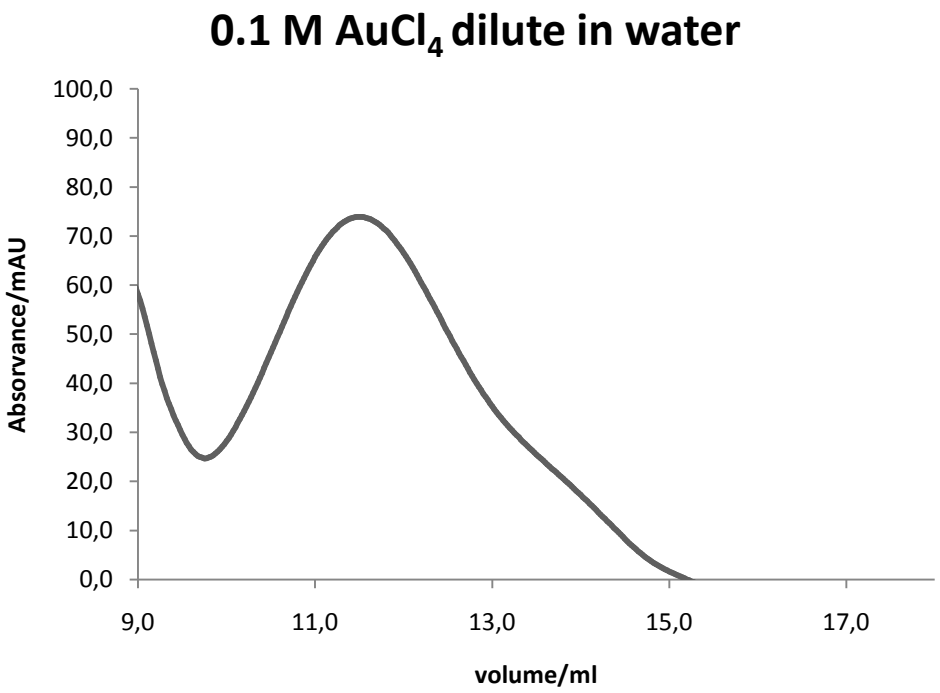

Figure 26- SEC of BFR in 0.01M AuCl4 dilute in water with AuNPs integrated

Here we can see a peak at around the correct cage elution volume which is red in color. Both signs that the particles have been formed and that they can of the correct size as dictated by the cavity size. However TEM analysis in figure 27, while showing dots with halos, more indications of encapsulated nanoparticles, does not show empty ferritin cages. This process is far from 100 % efficient, so the presence of empty cages is a key to the analysis as to whether or not an experiment has worked.

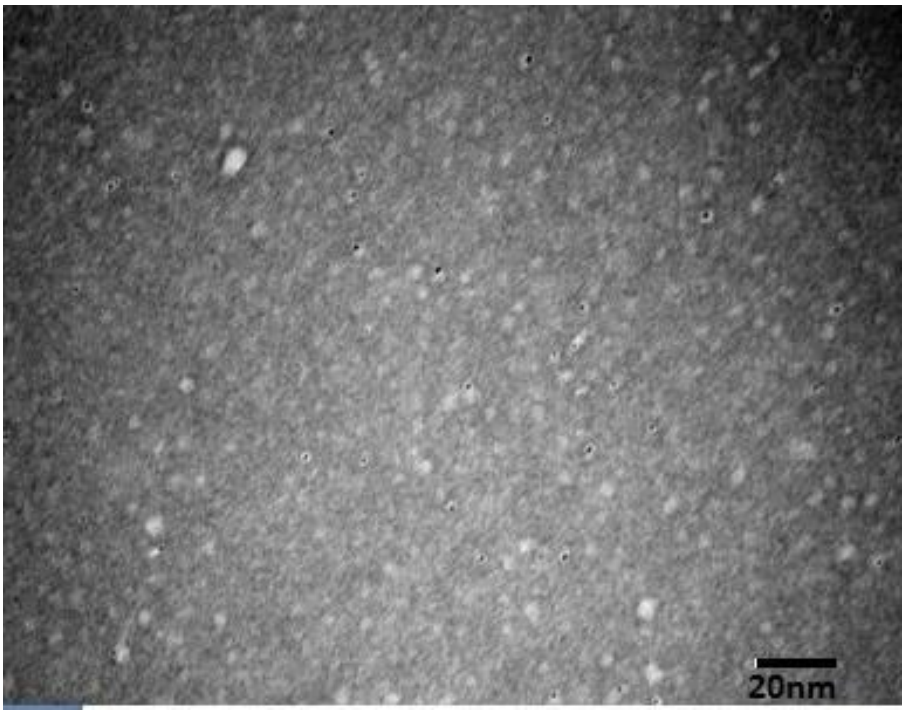

Figure 27- TEM image AuNPS in BFR; TEM: HV:120kV

The nanoparticles obtained are monodispersive and spherical. The average diameter is 6nm. Is possible to observe gold nanoparticles (black circle) surrounded by a white circle. However is not certain that the white circles are the ferritin, BFR.

To full analyze the formation of nanoparticles in BFR, similar experiment were set up to those used for DPS

Survability test for the incubation of gold in BFR

For BFR the same survability tests were performed as for DPS, in the presence of $AuCl_4$.

In table 3 is shown the results obtained for these test.

Table 3- Survability test of BFR in $AuCl_4$

0.01 M $AuCl_4$dilute in water	DPS cage peak no observed
0.01 M $AuCl_4$dilute in water added slowly over 30min	DPS cage peak no observed
0.1 M $AuCl_4$dilute in TRIS	DPS cage peak observed
0.01 M $AuCl_4$dilute in TRIS	DPS cage peak observed
0.01 M $AuCl_4$dilute in TRIS added slowly over 30min	DPS cage peak observed

The elution volumes of the experiments were obtained by SEC and are showing in figure 28,29,30,31 and 32 respectively.

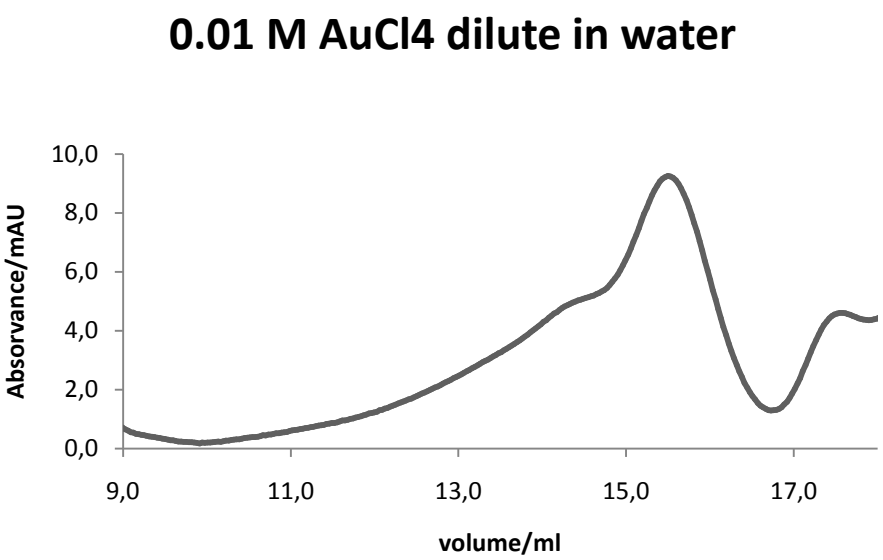

Figure 28-- SEC of BFR in 0.01 M $AuCl_4$ dilute in water

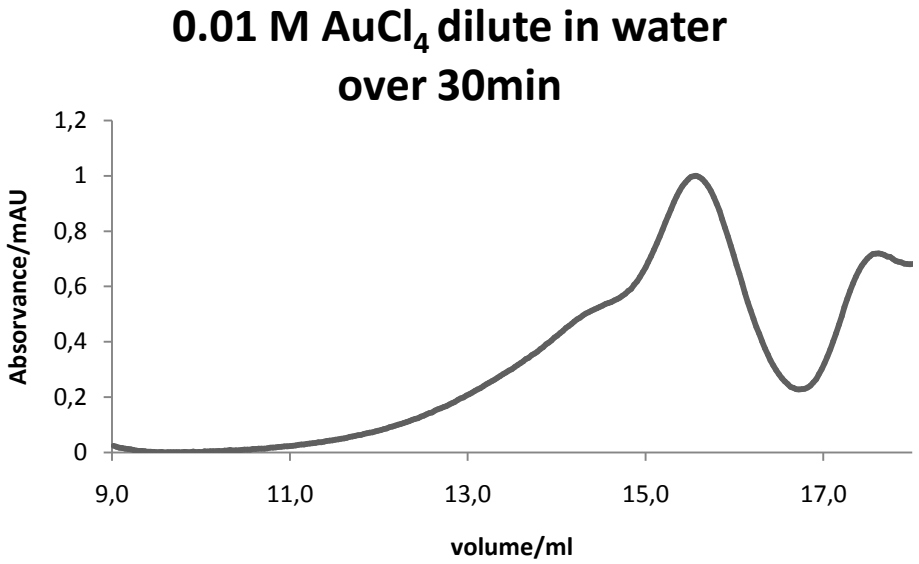

Figure 29- SEC of BFR in 0.01 M AuCl4 dilute in water over 30 min

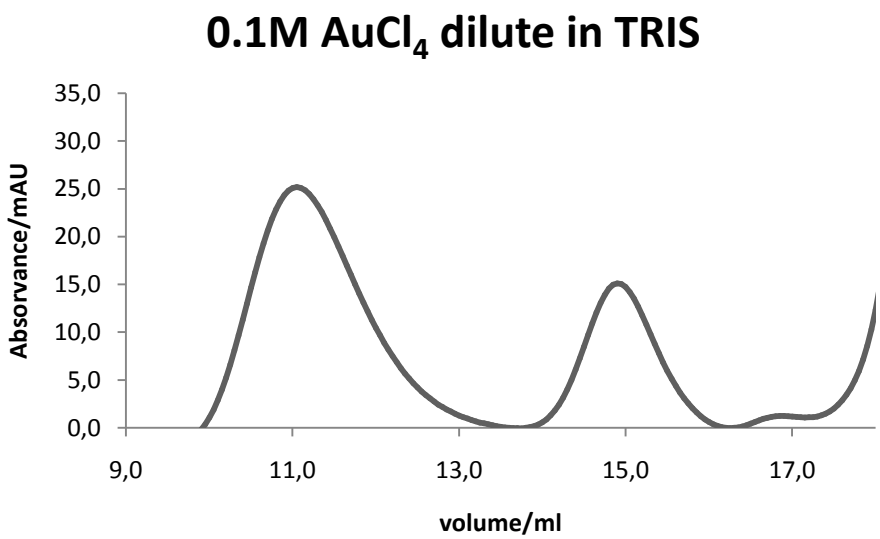

Figure 30- SEC of BFR in 0.1M $AuCl_4$ dilute in TRIS

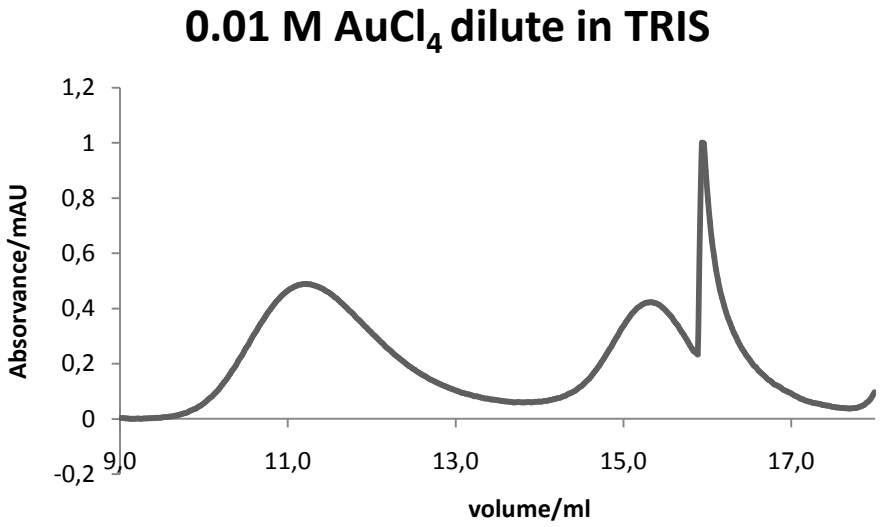

Figure 31- SEC of BFR in 0.01 M AuCl4 dilute TRIS

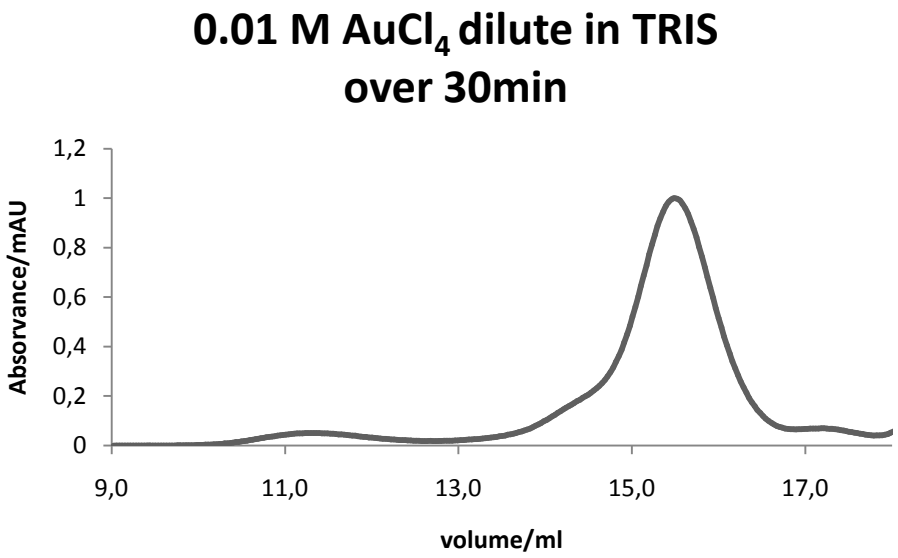

Figure 32- SEC of BFR in 0.01 M AuCl4 dilute TRIS over 30 min

Interestingly, the addition of TRIS and the increase in pH to 7.5 for the gold solution had the greatest effect on the survivability of the protein cage. Is also possible to see a complete loss of cage when this is performed with the same condition as the original method. This suggests that what is observed then was in fact an artifact.

Survivability test for the first reduction of gold in BFR

After testing the survability of BFR in $AuCl_4$ presence the next step was to produce gold nanoclusters inside of BFR.

Using the data obtained from the first step as a guide the second step was the analyse of this process, so further experiments were performed using 0.1M $AuCl_4$ in water with the results shown in table 4. However down to time constraints only the latter experiment was tried.

Table 4- Results for the production of gold nanoparticles inside BFR

0.1 M $AuCl_4$dilute in water	DPS cage peak observed
0.1 M $AuCl_4$dilute in TRIS	DPS cage peak observed

The elution volume of the experiment was obtained by SEC and is shown in figure 33. In figure 34 is shown the TEM image for this experiment.

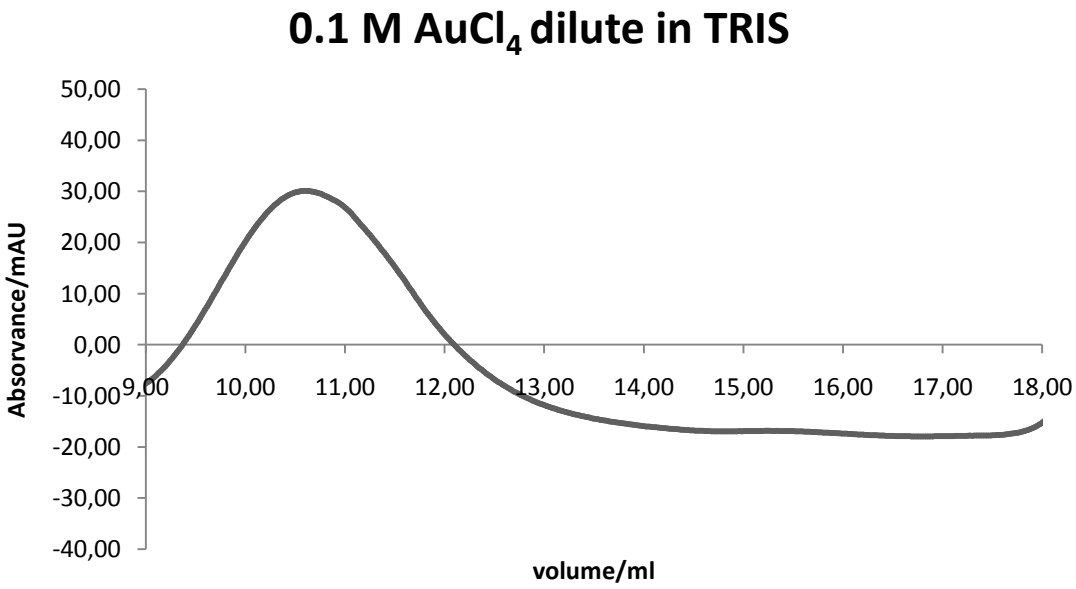

Figure 33- SEC of BFR in 0.1 AuCl4 dilute in Tris with AuNPs integrated

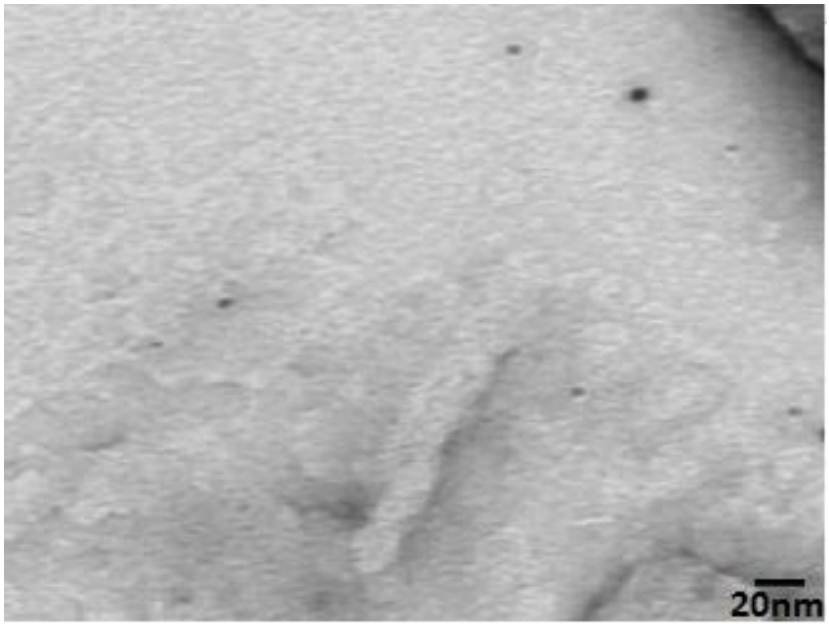

Figure 34-- TEM image of BFR in 0.01M AuCl4 dilute in TRIS
120kV

While the SEC shows a peak similar to that of BFR, the TEM analyse was again inconclusive. While nanoparticles of a relatively singular size were seen, no empty cages were present. This leads to several conclusions, 1) that while the process itself causes the ferritin cage to initially dissociate, the formation of nanoparticles might help to stabiles some, causing a size constraint vessel, but only in the presence of the nanoparticle. This would lead to what observe with little to no empty cages. 2) that the protein is completely denaturing and the white halo seen is just protein chains that have covered the nanoparticle surface. Their size might be a factor of the time allowed during this experiment that happens to produce nanoparticles of a similar size to the observed BFR in SEC.

5. Conclusions

The production of gold nanoparticles inside horse spleen ferritin is a well characterized method that could be very useful in future applications if it could be expanded. The ferritins BFR and DPS were purified and quantified using an FPLC (histrap and SEC) and BCA method. The published method for producing nanoparticles inside of unmodified ferritins did not provide data that corresponded to the results seen with HSFn. Instead of attemping to tackle the whole experiment, it was deconstructed it into its parts, 1) initial incubation with gold, 2) reduction into a nanocluster, after the initial incubation of gold, 3) the reconstruction of the whole experiment. These were analyzed by SEC and TEM to observe their outcome.

While not have time to complete the entire experiment for DPS, some progress has been made. Several methods were examined to find better ways to encourage protein stability while trying to create encapsulated nanoparticles. The change from water to TRIS, as a higher pH was a large step forward in keeping the protein stable. Also during the second step where the method attemps to creat a nanocluster, was also observed a cage peak. Bring this data back together presented new problems as ascorbic acid seemed to be playing a role in protein denaturation. After a complete change in reducing agents used, is started to see the presence of a protein peak at the end of the complete experiment. However, no nanoparticles were observed. While one challenge, the retention of protein cages has been moved forward, our new method provides an additional challenge in that the conditions might now be too mild for the creation of nanoparticles.

BFR provided a more complicated story. Some of the initial data suggests that the publish method had worked, but upon further investigation, it failed to survives. While there was some progress towards finding a method that allowed for the protein cage to remain intact, more time was needed.

5.1 Limitations and future work

While initial work has highlighted interesting new methods that can be used to help in protein survivability during this process, no concrete data was obtained that showed the encapsulation of nanoparticles by either DPS or BFR. In the future, the continues work with DPS needs to be made to take advantage of the knowledge gained from the first two steps. By reconstructing the whole experiment using some of these new methods maybe would be possible to start to see nanoparticles and intact ferritins.

Other methods could be used to confirm that the gold nanoparticles are integrated in the ferritins such as SDS polyacrylamide gel, the native polyacrylamide gel to help shed light on what is happening to BFR. Another method that could be made is the sucrose gradient, this method will remove the protein aggregations given a better image of the empty cells and the ones with encapsulated nanoparticles.

5.2 Final appreciation

The elaboration of this work represents for the author an exciting challenge and a test for their capabilities. This project given the opportunity to acquire knowledge in analytical chemistry specially in the FPLC machine.

6. References

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7. Additional information

Appendix A

Desalting graphs

In figure A.1, A.2 and A.3 are shown the desalting graphics made in the experiments for the different ferritins.

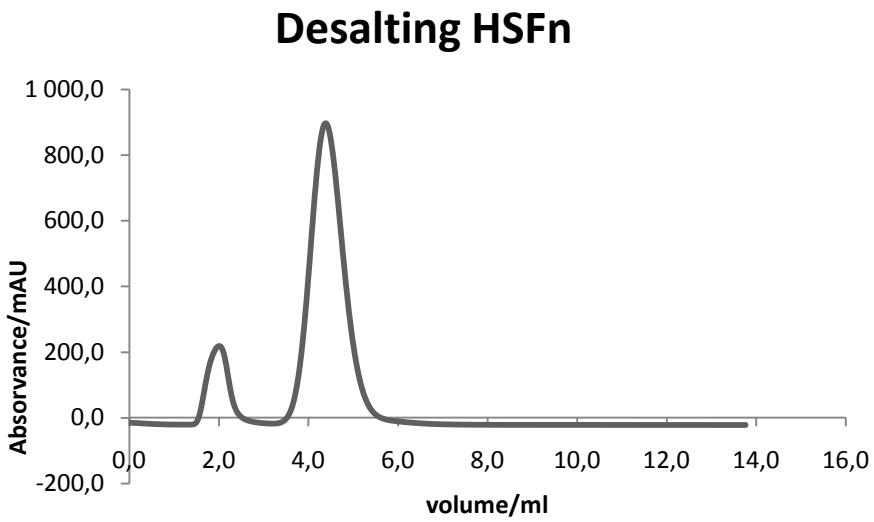

Figure A.1- Desalting for HSFn

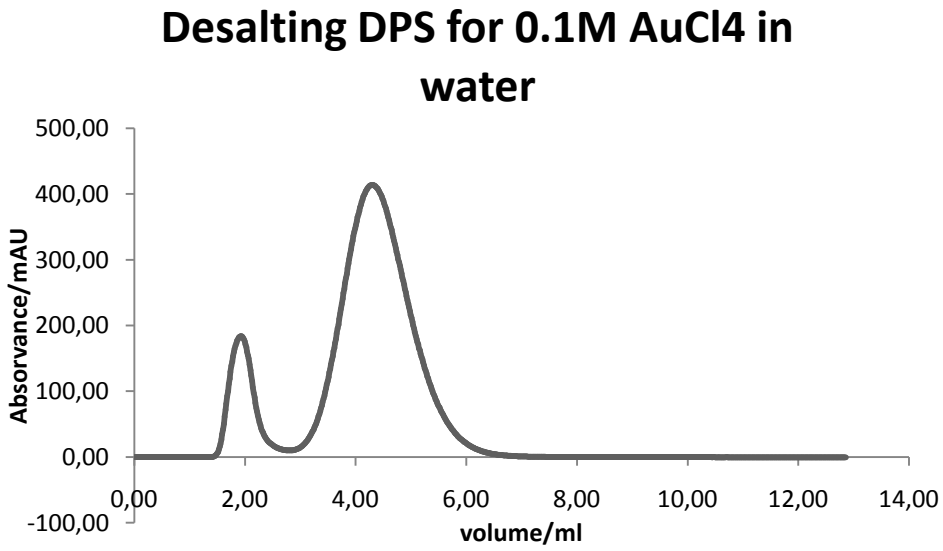

Figure A.2- Desalting for DPS

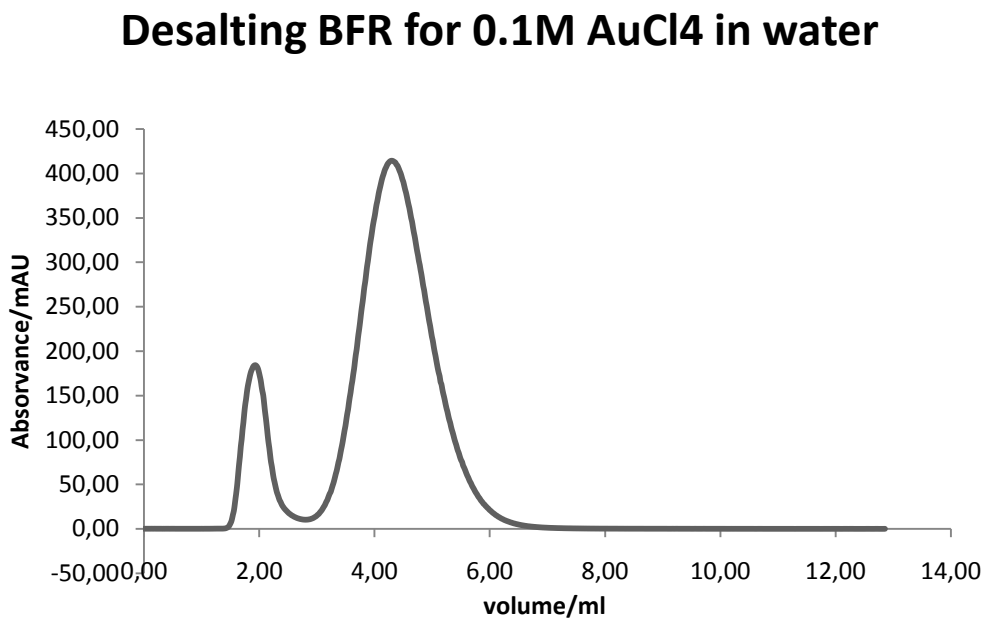

Figure A.3- Desalting for BFR